VARIATIONS OF THE SUBMICROSCOPIC STRUCTURE OF THE CORTICAL LAYER OF FERTILIZED AND PARTHENOGENETIC SEA URCHIN EGGS

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Introduction

In previous papers one of us (A. Monroy and A. Monroy Oddo, 1945, 1946) has investigated by means of polarized light the submicroscopic structure of the cortical layer of the unfertilized sea urchin egg. The cortical layer shows positive birefringence and normal optic axis, and since, in all the experiments made, no data came out suggesting that proteins enter into its constitution, Monroy and Monroy Oddo maintained that it is entirely of lipidic nature. According to its behavior in some experimental conditions, they considered it as a fluid crystal of the smectic type, which can be brought into the scheme of Bungenberg de Jong's complex ionic systems. However, according to Runnström and collaborators (1943–45), whose paper we were able to see only after the publication of Monroy and Monroy Oddo's note, the proteins also take part in the constitution of the cortical layer of both the unfertilized and fertilized sea urchin egg. This question will be discussed in a forthcoming paper.

We deal here with some results obtained by studying the variations of the submicroscopic structure of the cortical layer of the fertilized or parthenogenetically activated egg. Monroy (1945) has already pointed out some rhythmical variations of the cortical birefringence after fertilization, up to the two blastomere stage. We thought it worthwhile to investigate more accurately the rhythms of the observed variations, putting them in relation with the process of mitosis and with the modifications of permeability which can be observed in the fertilized and parthenogenetic egg.

A short note on these findings has already been published in *Nature* (Aug. 17, 1946).

Experiments on Fertilized and Parthenogenetically Activated Eggs

Observations and experiments were carried on in February–April, 1946, at the Zoological Station, Naples, on eggs of *Psammechinus miliaris* artificially fertilized or activated by Loeb's method. For this purpose, the unfertilized eggs were treated with a solution of 2.8–3.2 cc. of N/10 butyric acid in 50 cc. sea water. After washing in slightly alkaline sea water, they were observed in pure sea water.

During the first series of experiments the temperature of the water was 13°–15° C., in the second series (April), 17°–19°. The developmental cycle was of course remarkably accelerated by the rise of temperature: in the first series the first cleavage of the fertilized eggs and the climax of the first monastral cycle in the parthenogenetic eggs took place after 1 hour and 30 minutes, in the second series after one hour.

Birefringence of the cortical layer

Fertilized eggs. At fertilization the cortical birefringence of the unfertilized egg disappears, while in polarized light the egg surface acquires a slight diffuse luminosity (Fig. 3). After ca. 15–20 minutes, when the sperm-aster is in course of development, a slight silver-white positive birefringence appears in the cortical layer. In a short time its intensity increases (although it never reaches the intensity it has in the unfertilized egg, and remains always white) and later on it decreases and disappears before the total regression of the sperm-aster. From the very first series of experiments, we noticed a somewhat irregular behavior of this first birefringence cycle; its relation to the sperm-aster cycle was not constant, in some cases its appearance being earlier, in others later than the beginning of the sperm-aster.

In the second series of experiments, which we performed at a higher temperature, the first birefringence cycle was even more irregular. In some lots we could not see it, in others it appeared irregularly and for a short time only in some of the eggs, usually before the sperm-aster was visible. We shall discuss the possible meaning of this behavior later, in connection with the experiments with hypertonic solutions.

A second cycle of cortical birefringence, also with positive character, extends from the metaphase to the telophase of the first mitosis, as already described by Monroy (1945). It is characterized by a much higher intensity of the birefringence, which sometimes reaches the same level as in the unfertilized egg. Furthermore, it is absolutely constant under our experimental conditions.

As soon as the first cleavage is completed, the cortical birefringence disappears. It appears again during the ana-telophase of the second mitotic cycle.

Parthenogenetic eggs. In the eggs kept in butyric acid solution, the birefringence becomes more or less white, decreases in intensity and sometimes disappears. After ca. 10 minutes, if the eggs are replaced in sea water, it resumes the intensity and the color of the unfertilized egg, and remains so indefinitely (observations up to one hour after treatment).

The eggs removed from the butyric acid solution after a few minutes and replaced in sea water are activated. A variable percentage, in the various lots, elevate the membrane, while other eggs show incomplete membranogenesis or a total failure of it, depending on individual differences between the females used and on slightly different concentrations or exposure times. The eggs with no membranogenesis, however, are also activated as appears from the nuclear and monastral cycles. It is known in fact, that in the eggs simply activated and not subjected to the second step of Loeb's treatment (hypertony), successive monastral cycles develop, which finally lead to cytolysis.

In the activated egg, with or without a fertilization membrane, the cortical birefringence disappears as in the fertilized eggs. From 15 to 20 minutes later the first birefringence cycle appears and its behavior is entirely similar to that of the fertilized eggs. Also in this case, if the eggs are kept at a higher temperature, the first cycle is more irregular.

The first cycle comes to its end, and, about one hour after activation, the second cycle begins, showing the same characters of constancy and intensity as in fertilized eggs. When the monaster regresses, the cortical birefringence also fades away and

disappears. Half an hour later the second monaster cycle develops and the birefringence reappears. A third, fourth, fifth monaster cycle follows, with more and more accelerated rhythms, and the egg finally cytolyzes. We observed the reappearance of the birefringence up to the third monaster cycle, and we have little doubt that it appears also in the following.

To summarize, the observations of fertilized and parthenogenetic eggs in polarized light show that in both cases there exists a first cycle of cortical birefringence, rather labile, appearing ca. 15–20 minutes after fertilization or parthenogenetic activation. It soon fades away and the egg shows a slight diffuse luminosity, which is probably due to small granules. Almost synchronic with the maximum expansion of the diaster, or the monaster, the cortical birefringence reappears very intensely. This second cycle also fades away, and a third appears at the metaphase of the second mitosis or at the expansion of the second monaster in parthenogenesis. The birefringent layer is always immediately underlying the hyaline coat of the activated egg.

Experiments with hypertony

The permeability of the unfertilized, fertilized and parthenogenetic egg has been the object of very many researches and from the experiments of various authors it appears that after activation the permeability undergoes a series of cyclic variations. We will not summarize all the pertinent literature, referring for that purpose to the papers by Herlant (1920), Hobson (1932), Runnström (1923, 1929), Öhman (1945). We will take into account mainly the results of the last two authors. They have shown that the fertilized or parthenogenetic eggs, after treatment with hypertonic saline solutions immediately after activation and for a period of about 10 minutes, manifest a uniform total contraction, preserving an almost unaltered spherical form. To this phenomenon Runnström has given the name of "kugelige Plasmolyse" or spherical plasmolysis. Between 10 and 40 minutes after activation the egg reacts in a different way to hypertonic solution, showing a wrinkled surface, that is, the "eckige Plasmolyse" of Runnström, or angular plasmolysis. After ca. 50 minutes from activation, the spherical plasmolysis reappears, and lasts until the moment immediately preceding the first cleavage when the angular plasmolysis sets in. Runnström (1929) thinks that such phenomena are related to variations of the gelification of the cortical layer, the angular plasmolysis corresponding to a condition of greater rigidity.

We thought that a certain correspondence might exist between the type of plasmolysis and cortical birefringence. Therefore we repeated the experiments of the preceding authors, at the same time examining the eggs in polarized light. We used the same hypertonic solution as Runnström and collaborators, i.e., 20 cc. of sea water and 6 cc. of a 2.5 M solution of NaCl. The eggs were put into the solution at intervals of 5–10 minutes and then observed under normal and polarized light.

The unfertilized egg treated by this solution shows a "polyhedric" plasmolysis (Fig. 2) and loses its cortical birefringence. Immediately after fertilization or parthenogenetic activation, a strong spherical plasmolysis is observed while a superficial hyaline layer becomes visible. It contains small granules and is of variable thickness in different specimens. The underlying cortical layer proper shows a vivid birefringence with polarization cross. Such a condition lasts 10–

15 minutes (experiments made in April, at $t° = 17° - 19°$) and therefore it coincides, in part at least, with the first birefringence cycle observed in normal eggs. After this first period, the angular plasmolysis appears, while in polarized light no cortical birefringence with polarization cross can be detected. The egg surface, especially the edges, appears luminous because of the presence of rather intensely birefringent granules. Such a luminosity always occurs in the cortical layer proper, while the superficial coat, less clearly defined than in the spherical plasmolysis, does not show any luminosity. The angular plasmolysis begins when the evolution of the sperm-aster is in progress.

Such a condition lasts until the metaphase or expanding monaster stage. Then, ca. 50–60 minutes after activation, the eggs in hypertonic solution show again spherical plasmolysis with intense cortical birefringence (Figs. 5, 7, 8). The controls in normal sea water undergo the second birefringence cycle. In this phase the superficial coat in the normal egg has reached its maximum thickness. In eggs treated with hypertonic solution the coat shows an undulated contour, as if, being inelastic, it could not follow the egg mass in its contraction (Fig. 6).

We did not observe a reappearance of the angular plasmolysis shortly before the end of the division, as described by Runnström.

In the parthenogenetic eggs, during the monaster regression the angular plasmolysis reappears, while in the controls birefringence disappears. In the second, third, etc., monaster cycle the spherical plasmolysis reappears, always in association with birefringence.

To summarize: these experiments show that the two types of plasmolysis mentioned by the authors quoted above and observed again by us, correspond fairly exactly with the cycles of cortical birefringence; namely, spherical plasmolysis corresponds with the phases of evident cortical birefringence, angular plasmolysis corresponds with the phases of lacking birefringence. We should like to point out again the fact, which will be discussed later, that during the first 10–15 minutes after activation, the cortical birefringence of the controls, at the conditions of temperature stated above, does not always manifest itself clearly. The hypertonic treatment, on the contrary, makes it very striking in all the eggs.

We also noticed that hypertony makes more evident a birefringence of the asters, already described by Schmidt (1939) and Monné (1945) (Figs. 5, 7, 8).

PLATE 1

All the figures are photographs of *Psammechinus miliaris* eggs at a magnification of 500 ×.

FIGURE 1. Unfertilized egg (polarized light): note the cortical birefringence.

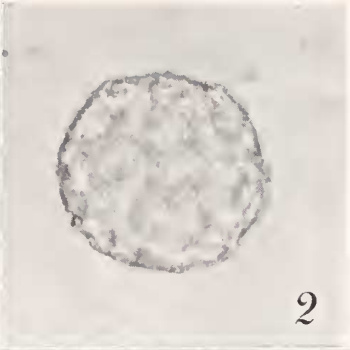
FIGURE 2. Unfertilized egg in hypertonic sea water (ordinary light): note the "polyhedric" plasmolysis.

FIGURE 3. An egg soon after fertilization (polarized light): note the birefringence of the fertilization membrane and the disappearance of the cortical birefringence.

FIGURE 4. An egg in the late metaphase stage (polarized light): the birefringence of the cortical layer is again evident.

FIGURES 5 AND 6. An egg in the late metaphase stage in hypertonic sea water as seen in polarized light (Fig. 5) and in ordinary light (Fig. 6). Note the smooth surface of the egg exhibiting a very brilliant birefringence (Fig. 5) and the wrinkled hyaline layer which does not exhibit any birefringence (Fig. 6). Note also the birefringence of the spindle as seen from a pole.

FIGURES 7 AND 8. Eggs in the beginning (Fig. 7) and late telophase (Fig. 8) in hypertonic sea water (polarized light): birefringence of the cortical layer and of the spindle-figure.



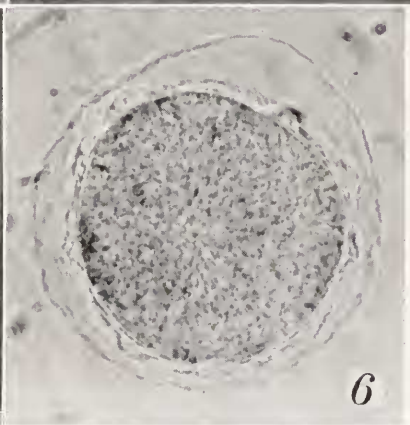


PLATE I

Colchicine experiments

The synchronism of the birefringence and the diaster and monaster cycles (in fertilized and parthenogenetic eggs respectively) as well as of the plasmolysis cycles leads one to think that all these phenomena are linked with one another. We were able to observe, however, that the first birefringence cycle (15–20 minutes after

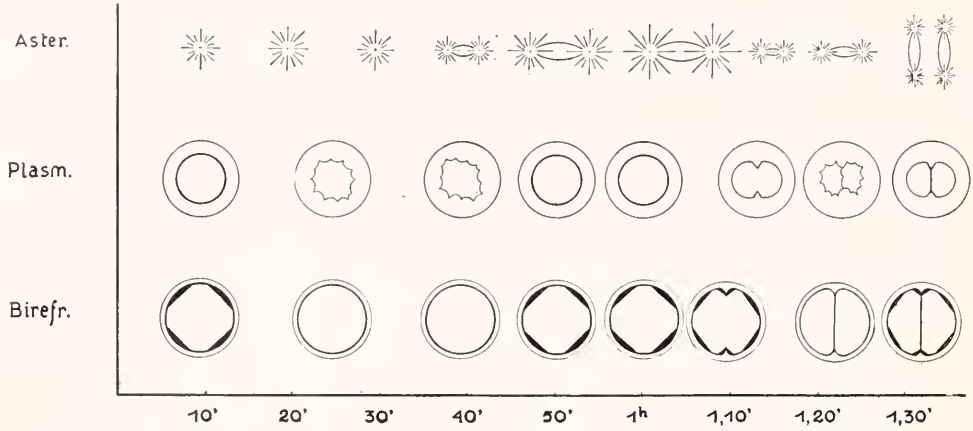


FIGURE 9. Diagram showing the time correspondence between the cycle of the cortical birefringence compared with the development of sperm-aster and spindle and the types of plasmolysis in fertilized eggs.

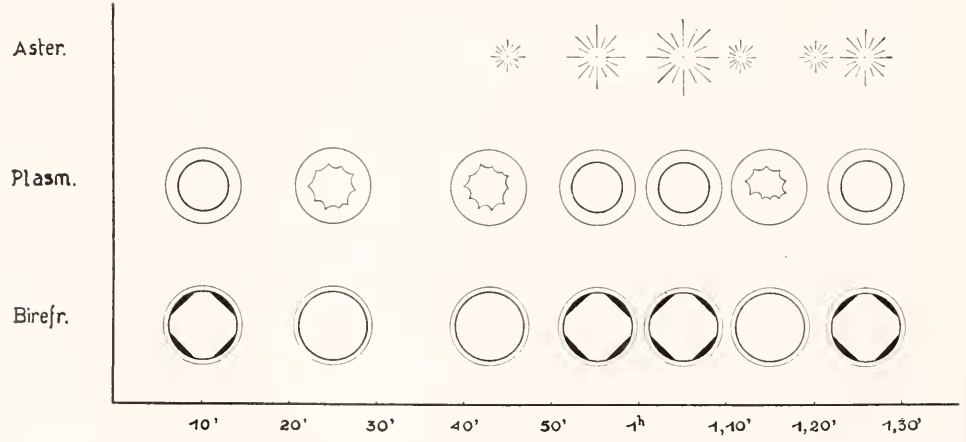


FIGURE 10. Diagram showing the time correspondence between the cycle of the cortical birefringence compared with the development of monaster and the types of plasmolysis in eggs activated with butyric acid.

fertilization) is not strictly correlated with the development of the sperm-aster, and in the parthenogenetic eggs, of course, is not connected with any phenomenon comparable to it. In order to test any possible correlation between the cortical birefringence and the aster cycles, we have treated the eggs with colchicine, with the aim of suppressing the aster.

The technique was the same as that used by Beams and Evans (1940), i.e., immersion of the fertilized or parthenogenetic eggs in a 0.0002 M solution of colchicine. In both series of experiments we observed the birefringence cycles, as already described, in complete absence of asters. The results are extremely clear cut, and therefore we can conclude that the cycles of the cortical birefringence, although normally synchronous with the cycles of the asters, are independent of them.

Furthermore the cycle of the two types of plasmolysis in colchicine-treated eggs proceeds exactly in the same way as in the controls. It is thus clear that the suppression of the aster by colchicine does not alter in any way the conditions of permeability of the cortical layer. Hence these experiments confirm Runnström's idea that the two types of plasmolysis are related to different conditions of the cortical layer only and not of the whole cytoplasm.

Conclusions and Discussion

From the above described investigations the following conclusions can be drawn:

1. The cortical layer of the unfertilized egg of *Psammechinus miliaris* shows a positive birefringence of yellow-orange color (Fig. 1).

2. Such birefringence disappears at fertilization or parthenogenetic activation (Fig. 3).

3. From 15 to 20 minutes after fertilization or activation, in certain conditions, a slight positive birefringence appears, of silver-white color. Hypertonic treatment makes it evident and constant also in cases in which in the controls it is faint or absent.

4. From 20 minutes to the end of metaphase, the cortical layer does not show any birefringence either in controls or in hypertonic treated eggs.

5. Beginning with the end of metaphase in fertilized eggs and with the starting of the monaster expansion in parthenogenetic eggs, a strong positive cortical birefringence appears, silver-white in color and very brilliant both in controls and in hypertonic treated eggs (Figs. 4, 5, 7, 8).

6. These cycles of variation of the submicroscopic structure of the cortical layer correspond exactly to the cycles of spherical and angular plasmolysis described by Runnström.

7. Aster inhibition by colchicine does not alter the cycle either of the cortical birefringence or of plasmolysis. The correspondence of the cycles of cortical birefringence, of sperm-aster and spindle in fertilized eggs, of monaster in parthenogenetic eggs and of the type of plasmolysis is summarized in the diagrams (Figs. 9, 10).

It is thus evident that after fertilization the cortical layer of the sea urchin egg undergoes cyclic variations of its submicroscopic structure, which are revealed by polarized light. Runnström, Monné and Broman (1943), on the contrary, did not find any variations of the cortical birefringence after fertilization, although they were surprised by the fact that corresponding to the modifications observed by Runnström (1923, 1928) in dark field, no analogous variations could be detected in polarized light.

We have already stated that the cortical birefringence which reappears soon after fertilization is very variable in intensity and constancy in the different lots and under different experimental conditions. Hypertonic treatment, however, makes it clear and constant in every egg. Furthermore we have noticed that in some eggs, a few minutes after fertilization (4–5 minutes) and, even more frequently, after parthenogenetic treatment, a very labile and short lasting birefringence appears, which soon fades away. All this indicates, to our opinion, that the cortical layer, after normal or parthenogenetic activation, up to ca. 15–20 minutes, is in a condition of particular lability in its submicroscopic structure, which probably has a physiological correspondence in the peculiar susceptibility to hypotonic solutions pointed out by Just (1922). Since the hypertonic treatment makes the birefringence evident, it can be assumed that, as an effect of activation, at first there is an increase of the distance between the molecules of the cortical layer, which however do not lose their orientation. Hypertony, by causing a decrease of volume, determines the approach of the molecules, thus showing their orientation.

We are not able, at the moment, to give an interpretation of the intimate mechanism of such morphological modifications, but we want to draw attention to the remarkable physico-chemical modifications which, as is well known, occur soon after fertilization. We regard it as probable that an important feature possibly concerned with the molecular orientation of the cortical layer, should be the variation of the distribution of some electrolytes and particularly of Ca, which have been observed by many authors soon after fertilization, e.g., Mazia (1937), Örström and Örström (1942), A. Monroy Oddo (in press). In fact, the importance of bivalent electrolytes (especially Ca) on the molecular orientation and attraction on complex ionic systems is known (Bungenberg de Jong et al., 1935; 1938). A loss of Ca may thus determine a decrease of the effective attraction between the molecules in the ionic layer, and thus a condition of greater hydration of the whole system. In this connection it is also interesting to note Öhman's (1945) observation that after fertilization a decrease of the free cephalin occurs, which, according to the author, is becoming linked to the proteins. That means a variation of the relations between the various constituents of the system, and it is not improbable that this fact also plays a rôle in determining a rearrangement of the composition and structure of the cortical layer.

Attention should also be called to a fact which might have some importance for the analysis of the action of butyric acid in the activation process. Unfertilized eggs put into butyric acid show a decrease in the intensity of the cortical birefringence, which at the same time becomes whitish. After a certain time in the acid the birefringence returns to the same condition as in unfertilized eggs. The period of decreased intensity corresponds approximately to the optimum time for a successful activation.

Following the first phase the second succeeds, lasting from 15–20 minutes up to 50–60 minutes after activation, and during this phase the birefringence disappears and angular plasmolysis sets in. One can interpret this fact by assuming that after the phase of molecular orientation characteristic of the unfertilized and freshly fertilized egg, a phase of disorientation follows. At this time the egg appears diffusely luminous in polarized light, and this is probably due to the lack of normality of the optic axis of the cortical layer.

As to the correspondence which we found between birefringence and spherical plasmolysis, and respectively isotropy and angular plasmolysis, we are inclined to think that in the first case the cortical layer, being formed by radially iso-oriented molecules, undergoes uniform contraction due to hypertony, while in the second case, because of the disordered molecular orientation, the resistance of the various points of the cortical layer varies, and thus it reacts to plasmolysis not by a uniform contraction, but by formation of wrinkles (cf., also, Runnström, Monné and Broman, 1943).

It might seem strange that the reaction of the fertilized egg to hypertony, while its cortical layer has an orientated structure, is different from the reaction of the unfertilized egg, where the cortical layer also has an orientated structure apparently entirely similar. The unfertilized egg in fact, as we said, in hypertonic solution contracts with a "polyhedric" surface and loses its cortical birefringence. We have already mentioned the chemical and structural variations which probably occur in the cortical layer of the egg after fertilization and in particular the loss of Ca and perhaps also of some of its lipidic components (cephalin ?). This results in a condition of greater softness than in the unfertilized egg. In the latter, the Ca content of the whole egg and probably also that of the cortical layer is greater, and this causes a greater condensation of its molecules. This is probably the cause of a higher rigidity upon which the characteristic type of plasmolysis depends. It is not improbable, however, that in this phenomenon a rôle is played also by particular relations of the molecules of the cortical layer with the subcortical proteins, as the researches of Runnström, Monné and Broman (1943) seem to suggest. They found that trypsin-treated unfertilized eggs in hypertonic solution contract with a smooth surface, without losing their cortical birefringence.

Apparently this mechanical interpretation fits the observed facts better than Öhman's (1945) explanation. He considers the cortical layer as a lipo-proteic film, in which the quantity of proteins and lipids may vary. Of course our interpretation does not exclude the possibility that during the two phases, viz., orientation and disorientation, the relations between cortical lipids and subcortical proteins may also vary. Herlant's (1920) observations on the variations of susceptibility of the egg to fat solvents which occur between fertilization and first cleavage do not seem to coincide well with the rhythms of plasmolysis; they seem rather to indicate the existence of phases of greater and lesser susceptibility which are not easy to interpret because of their irregularity. At any rate, it would probably be worthwhile to repeat such experiments in connection with observations in polarized light, taking into account especially Bungenberg de Jong's researches on the mode of action of organic and inorganic compounds on complex ionic systems, and in particular on the influence they have on the length of the carbon chain.

It is perhaps pertinent to recall here an observation by Öhman (1945), viz., that the formation of bubbles by the action of heat is entirely inhibited soon after fertilization i.e., during the contraction phase. Later on the frequency with which eggs show bubbles gradually increases and reaches a maximum between 20 and 30 minutes after fertilization. Then another phase sets in, characterized by a lesser frequency of bubble formation (not total inhibition as soon after fertilization) which extends up to the anaphase of the first mitosis, at which time it increases again. The coincidence of this rhythm with the phases of cortical orientation and disorienta-

tion which we have described is very striking. The fact might possibly mean that the bubble formation is greatly facilitated by the molecular orientation of the cortical layer, as was to be expected on the ground of Bungenberg de Jong and Bonner's (1935) researches on bubble formation in drops of coacervates.

In conclusion we believe that the lipidic character of the cortical layer of the egg is not lost at fertilization, although rearrangements in its chemical composition and consequent variations of physical properties may well take place. Furthermore we recall that one of us (Monroy, 1945) has already pointed out that the cortical birefringence which reappears after fertilization, during the second cycle, has the same characters and the same quantitative value as in the unfertilized egg.

Furthermore the following fact found by us seems interesting: while birefringence conditions and type of plasmolysis are undoubtedly linked to one another, no linkage seems to exist between these phenomena and the aster cycles, although some synchronism is undeniable. The colchicine experiments allowed us to demonstrate that the birefringence-permeability cycle can proceed entirely undisturbed when asters are inhibited. It is also a remarkable fact that in parthenogenetically activated eggs, at the end of the monaster cycle, angular plasmolysis reappears as in fertilized eggs at the end of cleavage. Clearly enough we are dealing here with a rhythm characteristic of the activated egg independent of cellular division.

Our researches have not thrown light on the submicroscopic structure of the superficial hyaline layer, which, as is well known, appears after fertilization and is more evident in hypertony. It did not show any birefringence in our experimental conditions. We were able to observe that the cortical birefringent layer is always underlying the hyaline coat (as already stated by Runnström, Monné and Broman, 1943) and that the latter, especially during the second phase of spherical plasmolysis, is apparently non-elastic, and therefore, being unable to follow the egg in its contraction, shows wrinkles and undulations. This observation seems to be in favour of the presence of some sort of cleavage between the hyaline coat and the cortical layer, and that would explain how E. B. Harvey (1934) was able to shift it entirely from the egg by means of centrifugation.

Summary

The authors have investigated the structural variations of the cortical layer of *Psammechinus miliaris* egg after fertilization or parthenogenetic activation, by means of polarized light, hypertonic treatment and colchicine.

They found regular cyclic variations of the birefringence of the cortical layer. A first inconstant cycle of birefringence appears at 15–20 minutes after fertilization or parthenogenetic activation. A second, more intense and constant cycle appears at the end of metaphase up to the telophase of the first cleavage, or, in parthenogenetic eggs, at the expansion of the monaster.

In eggs treated with hypertonic solution, "spherical plasmolysis" corresponds to the birefringent phases, while "angular plasmolysis" corresponds to the non-birefringent phases.

Colchicine, which inhibits the aster formation, does not alter either the birefringence or the plasmolysis cycles and their synchronism. The latter two phenomena are thus linked to one another, but independent of the cycles of the aster.

The meaning of the facts and their relations to the physico-chemical phenomena occurring during activation are discussed.

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